Report of the first Inter-Laboratory Comparison Test organised by the Community Reference Laboratory for Mycotoxins

Aflatoxins B_1, B_2, G_1 & G_2 in acetonitrile

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Summary

A proficiency test was conducted with 26 European National Reference Laboratories (NRLs) for mycotoxins. Test materials were aflatoxin stock solutions in acetonitrile with a previously certified content. Laboratories determined the aflatoxin content by liquid chromatography against their own standard solutions as reference by reverse-phase high-performance liquid-chromatography (RP-HPLC) with fluorescence detection. Laboratories used either commercially obtained standard solutions or gravimetrically prepared solutions from dry aflatoxin materials in combination with spectrophotometric confirmation of the content.
The overall reproducibility values (RSD_R) were 14.1%, 9.3%, 22.8% and 15.3% for Aflatoxin B_1, B_2, G_1 and G_2 respectively, reflecting the structure related sensibility of aflatoxins in solutions towards daylight and alkaline ambient. The precision figures reflect the variability of aflatoxin results that are solely related to currently applied calibration procedures.

Methodology

Aflatoxin calibrants were produced at the Reference Material (RM) Unit of the Institute fore Reference Materials and Measurements (IRMM) and are at the final stage of certification prior release as certified reference material (CRM). The content of these solutions has been established as given in Table 1.

Table 1: Certified values of aflatoxin calibrants

		µg/mL	MU
AC057	AfB1	2.97	0.09
AC058	AfB2	2.98	0.06
AC059	AfG1	2.96	0.1
AC060	AfG2	2.98	0.06

A full report on the production and certification of these solutions will be available upon the final certification of these materials by the RM-Unit of the IRMM.

Two calibrant ampoules for each aflatoxin (AfB_1, AfB_2, AfG_1 and AfG_2) were send to the participants as solutions of unknown concentration with a target range of 1 - 10 µg/mL. Participants were asked to measure a first set of four different ampoules on one day by triplicate analysis against their own standards and repeat the same experiment one week later with the 2nd set of ampoules. The instructions as send to the participants are added to the annex.
Results were reported by the participants via a web-interface. It unfortunately turned out that the web-interface which was made available to the CRL turned out to contain restrictions in the reporting of the dimension. Therefore participants were asked to report results as µg/mL regardless the dimension displayed during reporting. As some results had been already submitted when this limitation became evident, some results had to be normalised to reflect the correct dimension after data collection.

Results and Discussion:

Tables 1 to 4 list the single results submitted after dimension adjustment to µg/mL for day 1 (D1) and day 2 (D2) with the 3 replicates (R) for each day. Results that were reported as "0" or as "-" were excluded prior statistical analysis. In case of laboratory 109 only aflatoxin B_1 was measured the first day. In case of laboratory 121 no peak was identified for one aflatoxin B_2 ampoule.

Figures 1 to 4 show the sorted results from each laboratory for each toxin and Figure 5 the overall deviation from the certified content of the supplied standard, again sorted for each laboratory and by mean deviation. During the evaluation of results it could be observed that the bias of the replicates contributes mainly to observed deviation from the certified value.

Figure 5 gives a summary of the results from all four aflatoxins. This figure clearly indicates, that both effects, biased and random distribution related to the deviation from the certified value can be observed. Therefore both contribute to the rather wide spread of results. However, in those cases where a clear random deviation (without bias) from the certified value was observed for the reported aflatoxins from a laboratory, results showed that this deviation was generally of small magnitude.

For better visualisation of the desired effects, additional plots were made containing the relevant data for highlighting. Therefore figures 6 to 9 show the relative standard deviation obtained from all measurements reported for a single aflatoxin and therefore reflect the intermediate reproducibility sorted by single plots for each aflatoxin. Figures 10 to 13 show the absolute deviation in % of the mean value from the certified value sorted by single plots for each aflatoxin. Figures 14 to 17 are kernel density plots from all results reported sorted by single plots for each aflatoxin.

Table 1: Single results for different days (rounded to 3 significant figures) for aflatoxin B_1:

Laboratory code	D1R1	D1R2	D1R3	D2R1	D2R2	D2R3
101	2.76	2.73	3.20	2.70	2.67	3.21
102	2.47	2.21	2.18	1.96	1.87	1.92
103	3.10	3.10	3.00	3.03	3.09	3.13
104	2.87	2.84	2.84	2.69	2.71	2.72
105	3.37	3.54	3.81	2.79	2.43	2.50
106	3.10	3.15	3.16	3.12	3.11	3.15
107	2.83	2.79	2.79	2.77	2.78	2.78
108	2.86	2.85	2.95	2.93	2.88	2.83
110	2.78	2.89	2.79	2.76	2.95	2.83
111	3.05	3.04	2.99	2.97	2.94	3.00
112	2.29	2.26	2.30	2.69	2.67	2.67
113	2.76	2.76	2.76	2.82	2.82	2.85
114	2.90	2.86	2.92	3.09	3.13	3.11
115	2.50	2.48	2.46	2.20	2.21	2.19
116	2.62	2.60	2.63	2.78	2.72	2.74
117	2.80	2.89	2.80	2.95	2.87	2.92
118	3.71	3.59	4.07	4.06	4.06	4.16
119	3.04	3.06	3.06	2.97	2.97	2.97
120	2.69	2.69	2.66	2.23	2.26	0
121	3.04	3.03	3.05	3.05	3.05	3.05
122	2.90	2.98	2.93	2.94	2.95	2.95
123	3.13	3.12	3.11	3.00	2.97	3.01
124	3.47	3.70	3.75	3.50	3.64	3.70
125	2.99	2.96	2.77	3.11	3.26	3.16
126	2.48	2.48	2.47	2.49	2.48	2.49
109	2.27	2.25	2.28	-	-	-

Table 2: Single results for different days (rounded to 3 significant figures) for aflatoxin B_2:

Laboratory code	D1R1	D1R2	D1R3	D2R1	D2R2	D2R3
101	2.81	2.79	2.84	2.86	2.83	2.90
102	2.60	2.42	2.42	2.52	2.51	2.52
103	2.95	2.96	2.95	2.91	2.92	2.92
104	3.31	3.34	3.31	3.22	3.21	3.19
105	3.19	3.43	3.63	2.70	2.84	2.90
106	2.90	3.01	3.11	2.85	3.14	3.23
107	2.95	2.95	2.95	2.96	2.94	2.95
108	2.87	2.85	2.91	2.89	2.93	2.91
110	2.55	2.53	2.58	2.42	2.80	2.42
111	2.79	2.76	2.76	2.76	2.74	2.78
112	2.46	2.45	2.44	2.90	2.90	2.91
113	3.00	3.00	3.00	3.09	3.06	3.09
114	3.01	3.00	2.89	3.14	3.13	3.14
115	2.92	2.90	2.90	2.94	2.94	2.94
116	2.83	2.79	2.82	2.85	2.84	2.86
117	3.26	3.25	3.33	3.22	3.11	3.11
118	3.23	3.37	3.21	3.18	3.15	3.31
119	2.83	2.84	2.94	2.90	2.88	2.83
120	2.75	2.72	2.80	2.87	2.89	2.88
122	2.90	2.95	2.95	2.66	2.65	2.66
123	3.38	3.42	3.43	3.23	3.21	3.26
124	3.42	3.32	3.39	3.73	3.81	3.86
125	2.86	2.91	2.89	3.04	3.01	2.92
126	3.03	3.03	3.03	3.07	3.06	3.06
121	-	-	-	3.54	3.55	3.54

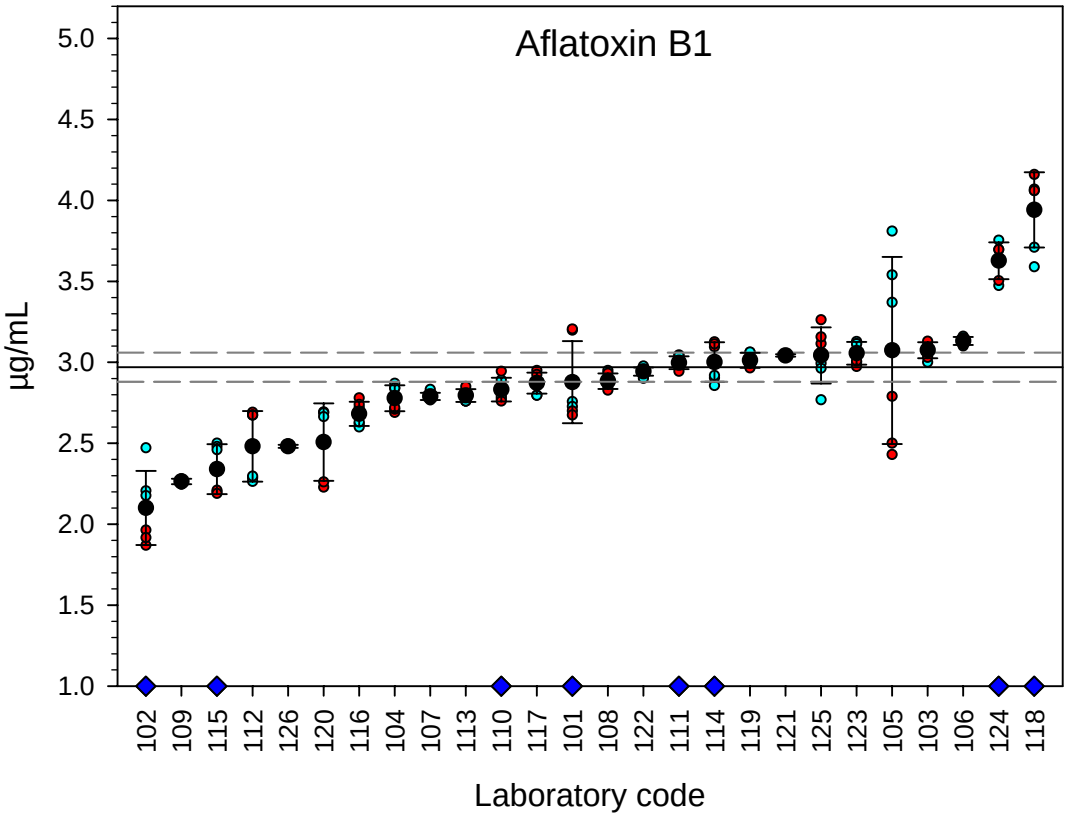
Table 3: Single results for different days (rounded to 3 significant figures) for aflatoxin G_1:

Laboratory code	D1R1	D1R2	D1R3	D2R1	D2R2	D2R3
101	2.84	2.84	3.47	2.89	2.87	3.51
102	1.23	1.27	1.28	1.19	1.25	1.26
103	3.17	3.16	3.11	3.26	3.22	3.23
104	4.17	4.16	4.17	4.06	4.07	4.05
105	3.10	3.20	3.03	2.44	2.48	2.54
106	3.00	3.16	2.97	3.19	3.23	3.18
107	2.87	2.88	2.85	2.90	2.93	2.95
108	2.72	2.75	2.73	2.82	2.82	2.83
110	2.86	2.86	2.86	2.88	2.93	2.86
111	2.88	2.91	2.88	2.81	2.83	2.84
112	2.39	2.38	2.42	2.77	2.77	2.77
113	2.97	2.94	2.97	3.03	3.03	3.03
114	3.04	3.10	3.01	2.89	2.91	2.95
115	2.12	2.12	2.08	2.52	2.53	2.53
116	2.61	2.56	2.58	2.68	2.66	2.68
117	0	3.79	3.75	4.03	4.02	3.95
118	4.37	4.53	5.02	4.02	4.30	4.24
119	3.12	3.16	3.14	3.02	3.07	3.05
120	2.79	2.75	2.75	2.47	2.48	2.48
121	4.01	4.02	3.96	4.31	4.26	4.30
122	3.08	3.04	3.05	3.07	3.07	3.08
123	3.56	3.53	3.51	3.32	3.27	3.32
124	3.95	3.98	3.99	4.28	4.37	4.38
125	3.02	2.93	2.88	2.85	3.05	3.04
126	2.23	2.23	2.23	2.31	2.29	2.29

Table 4: Single results for different days (rounded to 3 significant figures) for aflatoxin G_2:

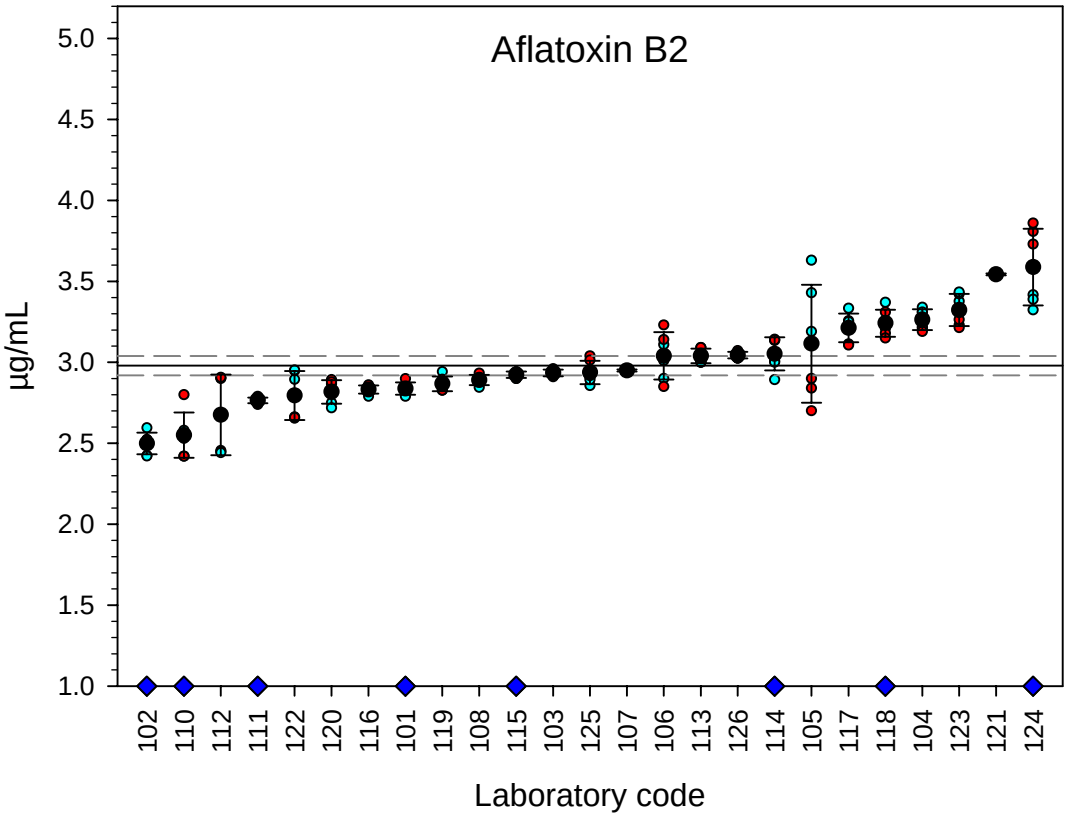
Laboratory code	D1R1	D1R2	D1R3	D2R1	D2R2	D2R3
101	2.96	2.95	2.98	2.96	2.96	3.01
102	2.89	2.86	2.89	2.82	2.83	2.85
103	2.69	2.70	2.68	2.79	2.71	2.72
104	2.66	2.66	2.68	2.70	2.72	2.72
105	3.42	3.77	3.45	2.98	2.92	3.08
106	3.09	3.08	3.02	3.03	2.97	3.03
107	3.02	3.03	3.02	3.03	3.04	3.01
108	2.80	2.80	2.78	2.91	2.91	2.89
110	2.87	2.89	2.95	2.82	2.88	2.86
111	3.78	3.68	3.68	3.73	3.81	3.73
112	2.57	2.58	2.57	3.01	3.02	3.04
113	3.00	3.00	3.00	3.06	3.06	3.06
114	2.96	2.91	3.07	2.70	2.70	2.71
115	2.98	2.98	2.94	2.95	2.98	2.95
116	2.92	2.93	2.91	3.11	3.12	3.09
117	3.66	3.98	3.90	3.81	3.86	0
118	3.19	3.35	3.22	2.98	3.10	3.23
119	3.07	2.95	2.98	3.06	3.01	2.99
120	0	2.95	3.01	3.03	3.06	3.05
121	4.81	4.81	4.81	4.96	4.97	4.98
122	3.21	2.99	3.12	3.13	3.14	3.15
123	3.99	4.01	3.99	3.59	3.59	3.64
124	3.51	3.39	3.48	3.73	3.77	3.75
125	3.02	3.20	3.03	3.08	3.07	3.12
126	2.92	2.93	2.95	2.95	2.94	2.93

Figure 1: Distribution of Results for Aflatoxin B_1



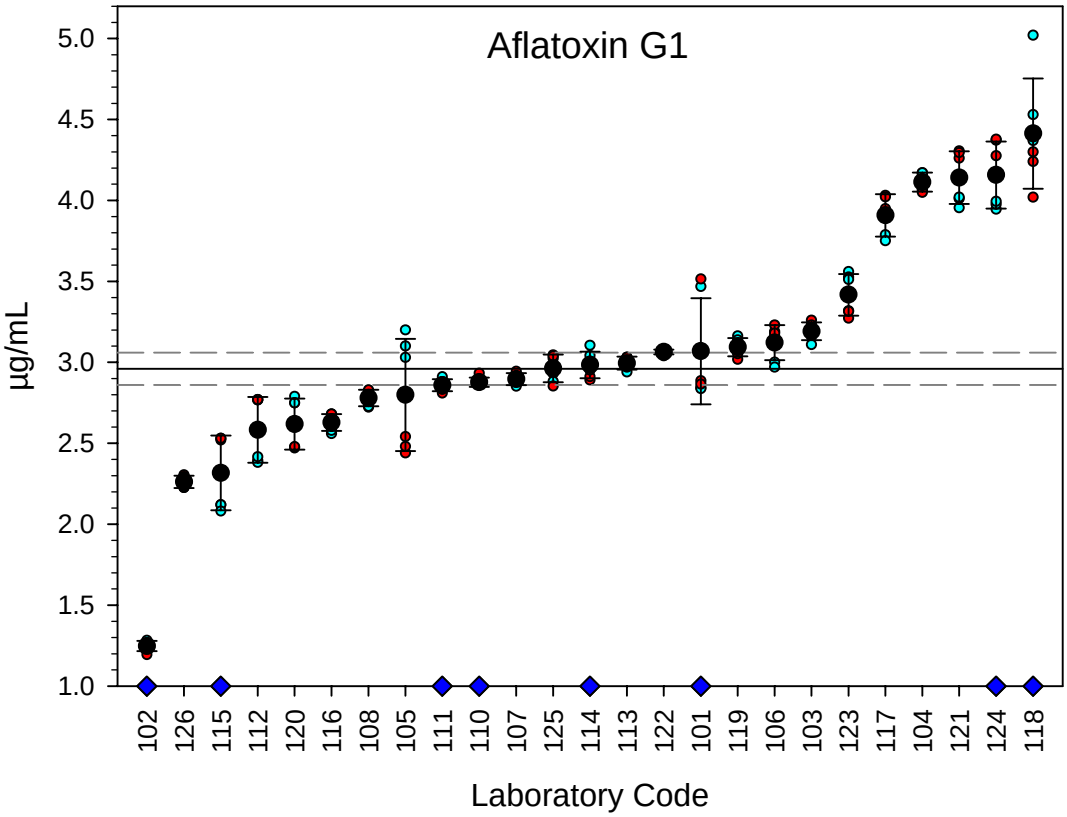
Laboratories highlighted with a blue diamond (◆) on the bottom line used commercially available standard solutions. In all other cases in-house prepared standards from dry material after spectroscopic determination of the content were used. Blue data points (●) are from the 1st day measurements, red data points (●) from the 2nd day. The black data point (●) represents the mean of all measurements from day 1 and day 2. The horizontal line indicates the certified value of the toxin with the stated uncertainty (dashed line). Vertical error bars indicate the standard deviation of the overall measurements.

Figure 2: Distribution of Results for Aflatoxin B_2



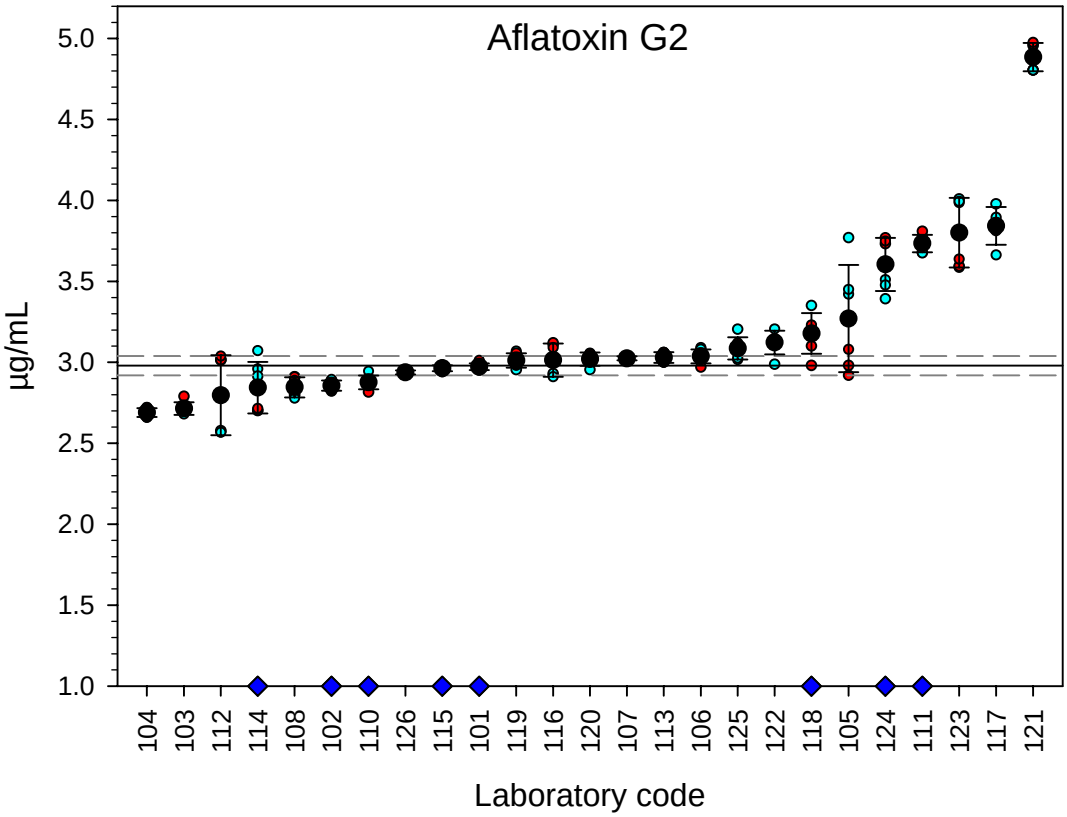
Laboratories highlighted with a blue diamond (◆) on the bottom line used commercially available standard solutions. In all other cases in-house prepared standards from dry material after spectroscopic determination of the content were used. Blue data points (●) are from the 1st day measurements, red data points (●) from the 2nd day. The black data point (●) represents the mean of all measurements from day 1 and day 2. The horizontal line indicates the certified value of the toxin with the stated uncertainty (dashed line). Vertical error bars indicate the standard deviation of the overall measurements.

Figure 3: Distribution of Results for Aflatoxin G_1



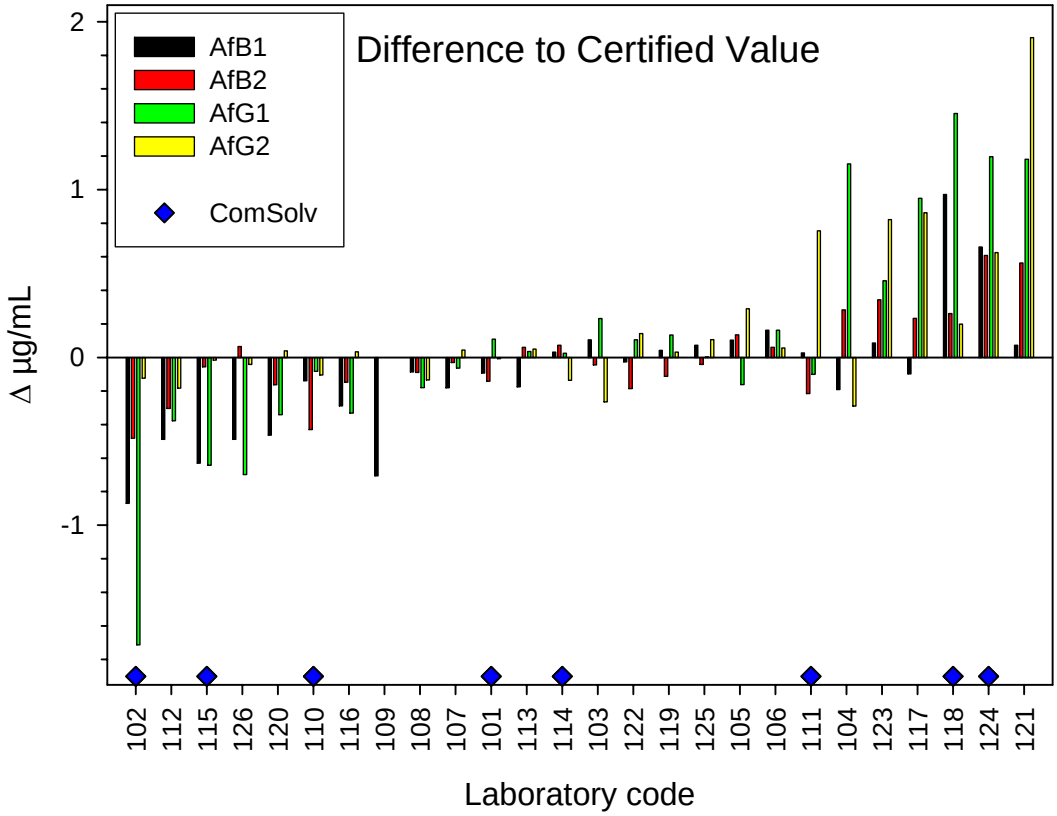
Laboratories highlighted with a blue diamond (◆) on the bottom line used commercially available standard solutions. In all other cases in-house prepared standards from dry material after spectroscopic determination of the content were used. Blue data points (●) are from the 1st day measurements, red data points (●) from the 2nd day. The black data point (●) represents the mean of all measurements from day 1 and day 2. The horizontal line indicates the certified value of the toxin with the stated uncertainty (dashed line). Vertical error bars indicate the standard deviation of the overall measurements.

Figure 4: Distribution of Results for Aflatoxin G_2



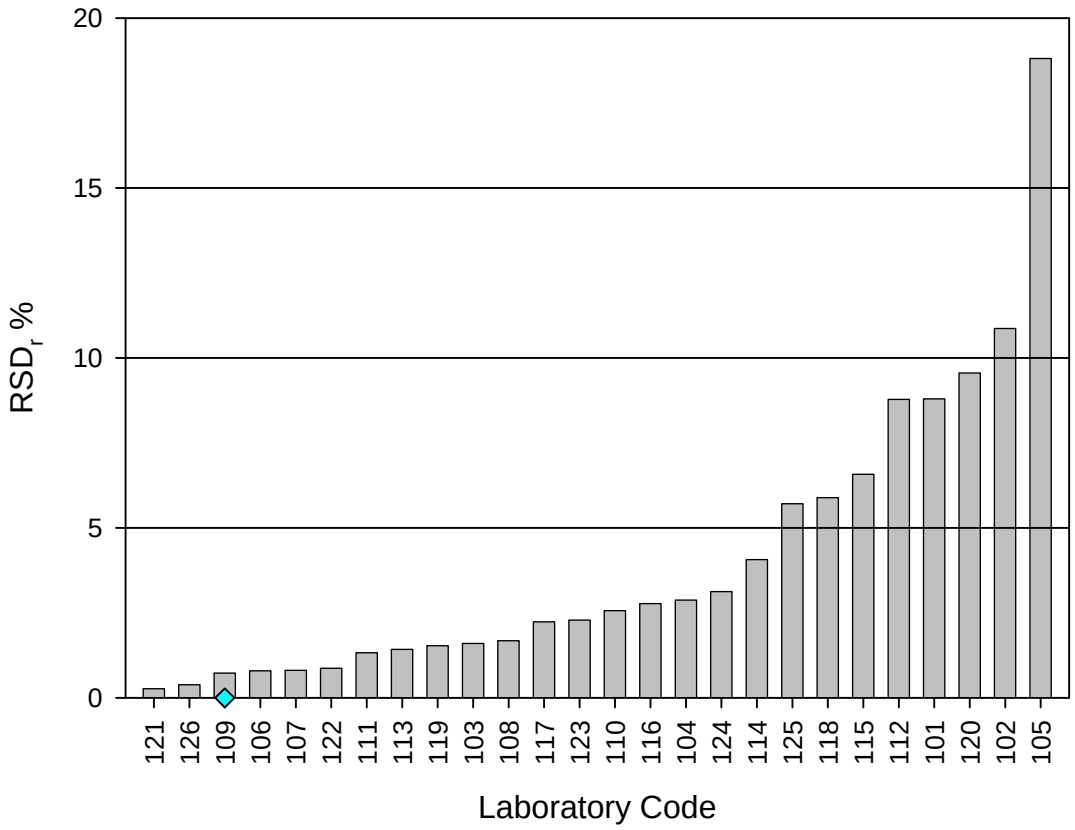
Laboratories highlighted with a blue diamond (◆) on the bottom line used commercially available standard solutions. In all other cases in-house prepared standards from dry material after spectroscopic determination of the content were used. Blue data points (●) are from the 1st day measurements, red data points (●) from the 2nd day. The black data point (●) represents the mean of all measurements from day 1 and day 2. The horizontal line indicates the certified value of the toxin with the stated uncertainty (dashed line). Vertical error bars indicate the standard deviation of the overall measurements.

Figure 5: Overall deviation from the certified content clustered by labs



Laboratories highlighted with a blue diamond (◆) on the bottom line used commercially available standard solutions. In all other cases in-house prepared standards from dry material after spectroscopic determination of the content were used. The bar length was calculated by the mean of all valid results from both days.

Figure 6: Intermediate reproducibility for aflatoxin B_1:



This figure shows the intermediate reproducibility of all submitted measurements from the first and the second day. The bar marked with a blue diamond (♦) is calculated only from measurements of one day (n=3). An intermediate reproducibility of less than 5% can be judged as good. Less than 10% are considered as acceptable for this exercise but should be improved. Values above 10% clearly show that laboratories need to undertake efforts to improve their measurements.

Figure 7: Intermediate reproducibility for aflatoxin B_2:

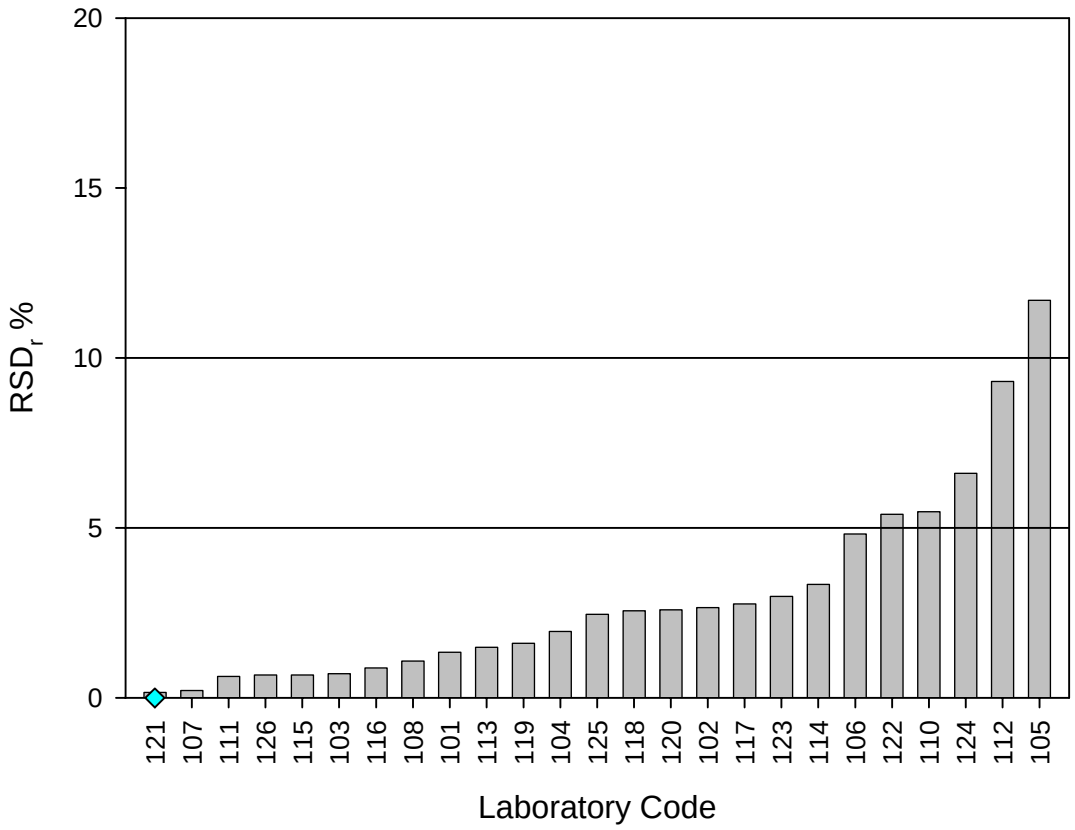


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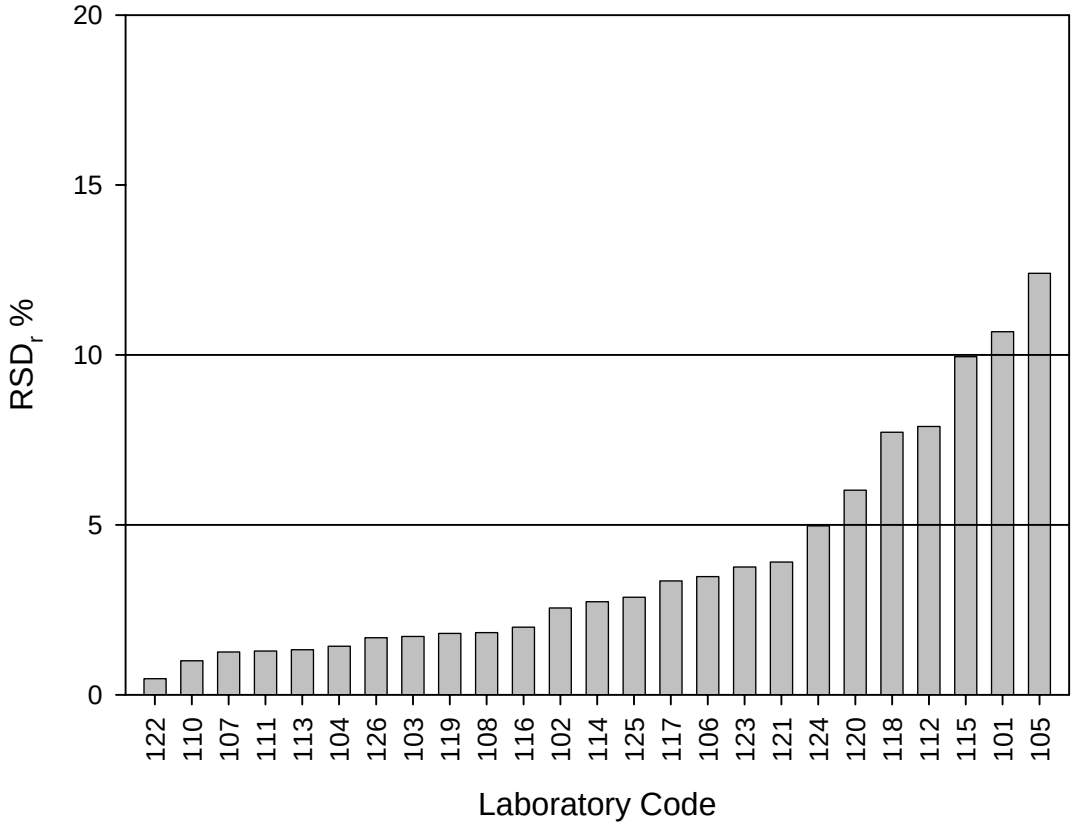
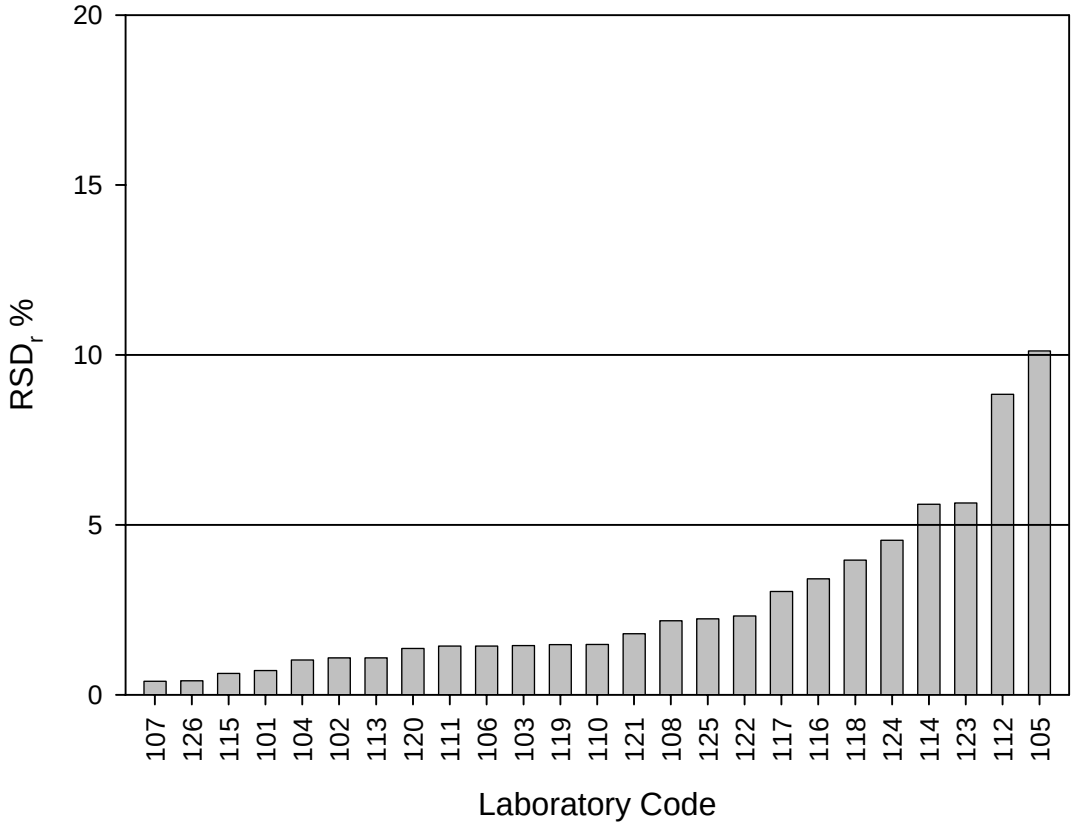
Figure 8: Intermediate reproducibility for aflatoxin G_1:



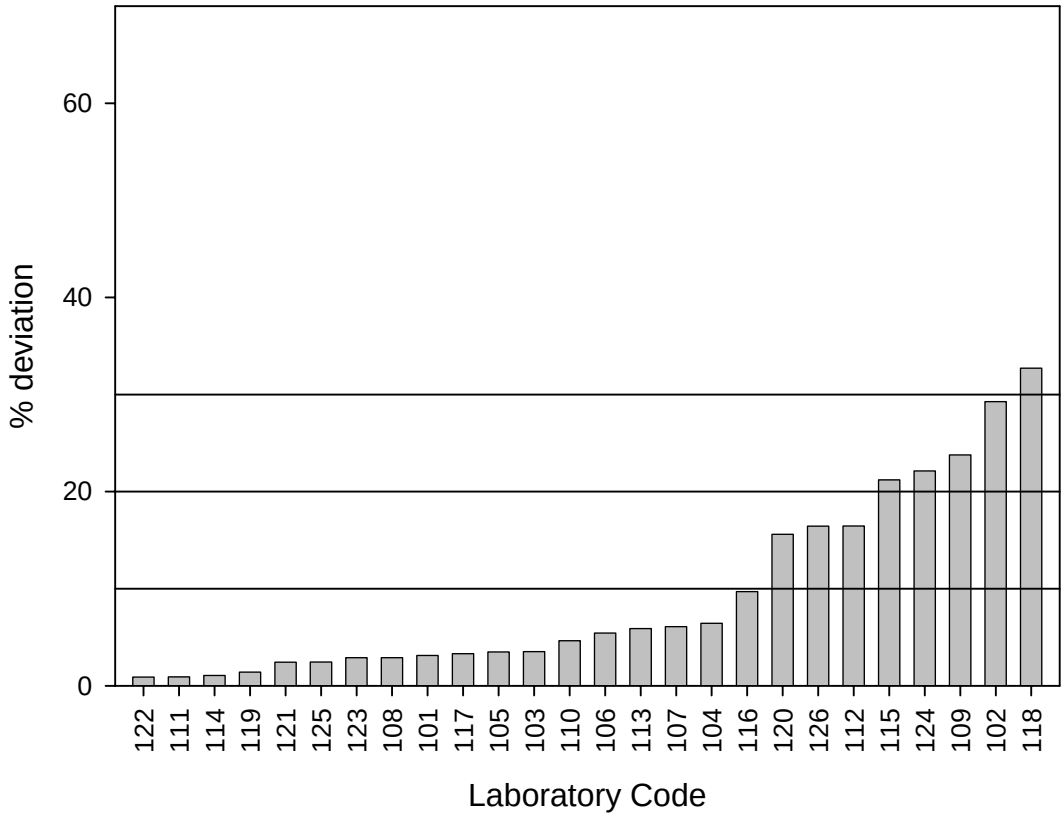
This figure shows the intermediate reproducibility of all submitted measurements from the first and the second day. An intermediate reproducibility of less than 5% can be judged as good. Less than 10% are considered as acceptable for this exercise but should be improved. Values above 10% clearly show that laboratories need to undertake efforts to improve their measurements.

Figure 9: Intermediate reproducibility for aflatoxin G_2:



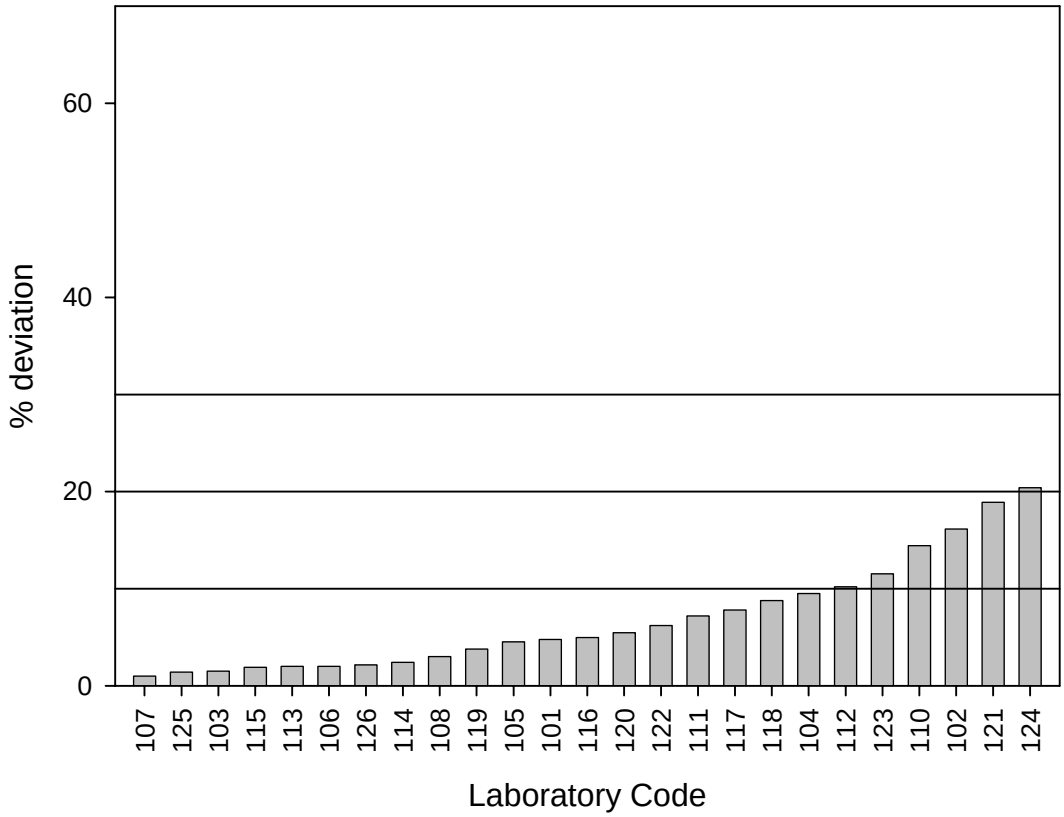
This figure shows the intermediate reproducibility of all submitted measurements from the first and the second day. An intermediate reproducibility of less than 5% can be judged as good. Less than 10% are considered as acceptable for this exercise but should be improved. Values above 10% clearly show that laboratories need to undertake efforts to improve their measurements.

Figure 10: Deviation of results from the certified value for aflatoxin B_1:



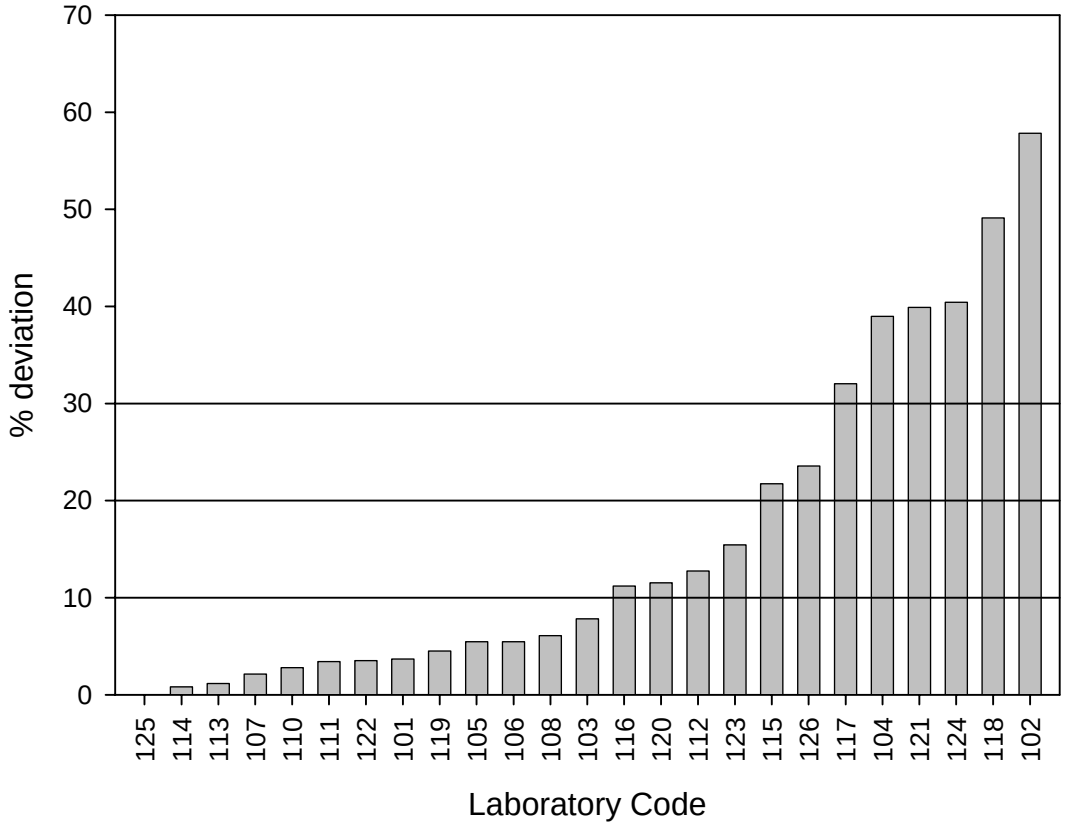
This figure shows the deviation of the mean result from all measurements from the certified value in percent (%). A deviation of less than 10% can be judged as sufficient. Values above 10% indicated that laboratories need to undertake efforts to improve their calibration procedure.

Figure 11: Deviation of results from the certified value for aflatoxin B_2:



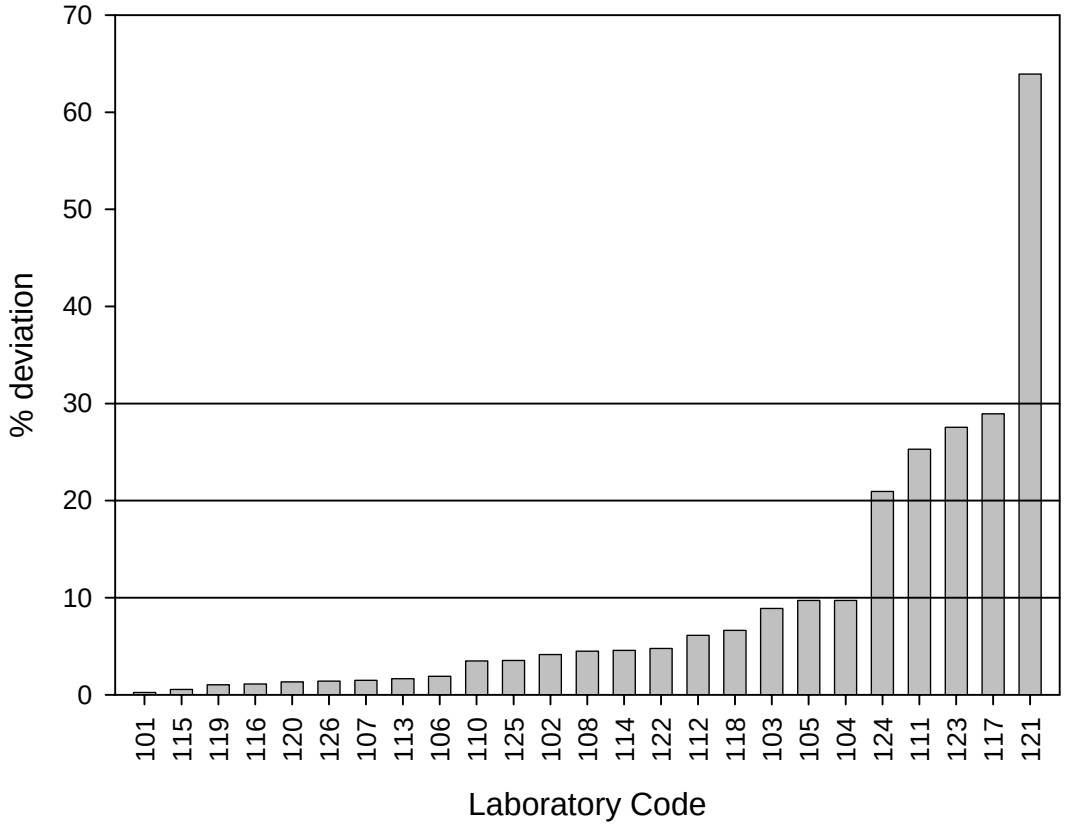
This figure shows the deviation of the mean result from all measurements from the certified value in percent (%). A deviation of less than 10% can be judged as sufficient. Values above 10% indicated that laboratories need to undertake efforts to improve their calibration procedure.

Figure 12: Deviation of results from the certified value for aflatoxin G_1:



This figure shows the deviation of the mean result from all measurements from the certified value in percent (%). A deviation of less than 10% can be judged as sufficient. Values above 10% indicated that laboratories need to undertake efforts to improve their calibration procedure.

Figure 13: Deviation of results from the certified value for aflatoxin G_2:



This figure shows the deviation of the mean result from all measurements from the certified value in percent (%). A deviation of less than 10% can be judged as sufficient. Values above 10% indicated that laboratories need to undertake efforts to improve their calibration procedure.

Figure 14: Kernel Density Plot for aflatoxin B_1 results:

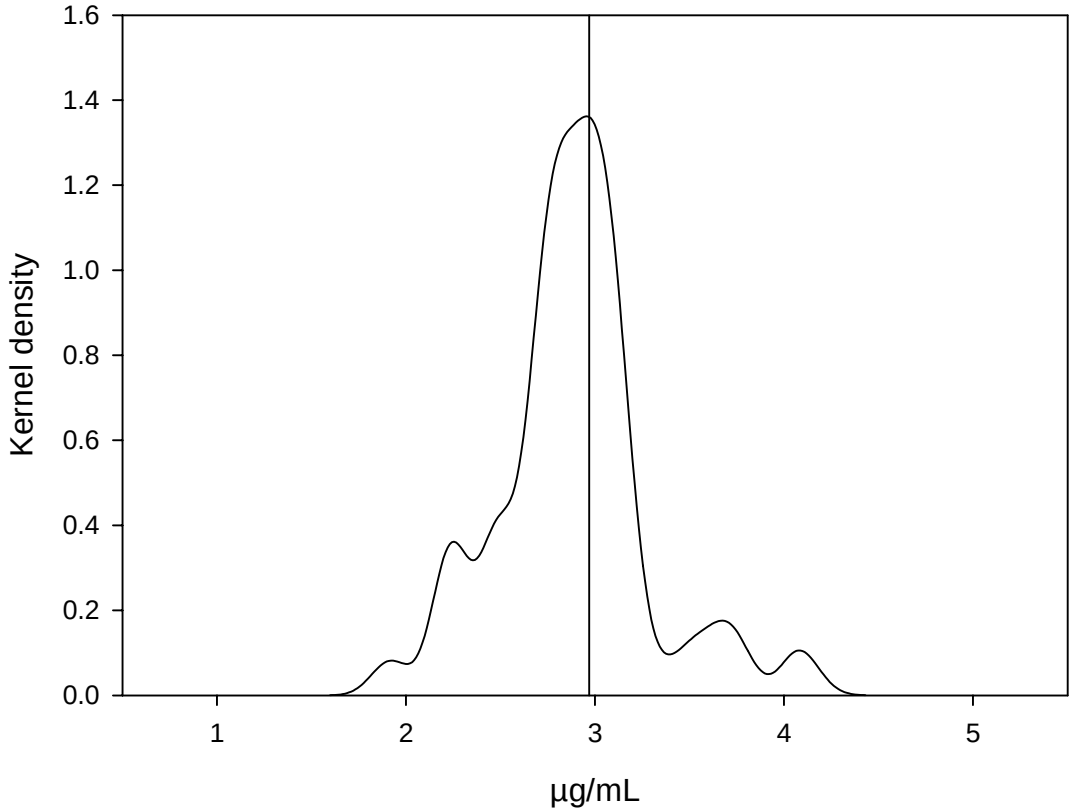


Figure 15: Kernel Density Plot for aflatoxin B_2 results:

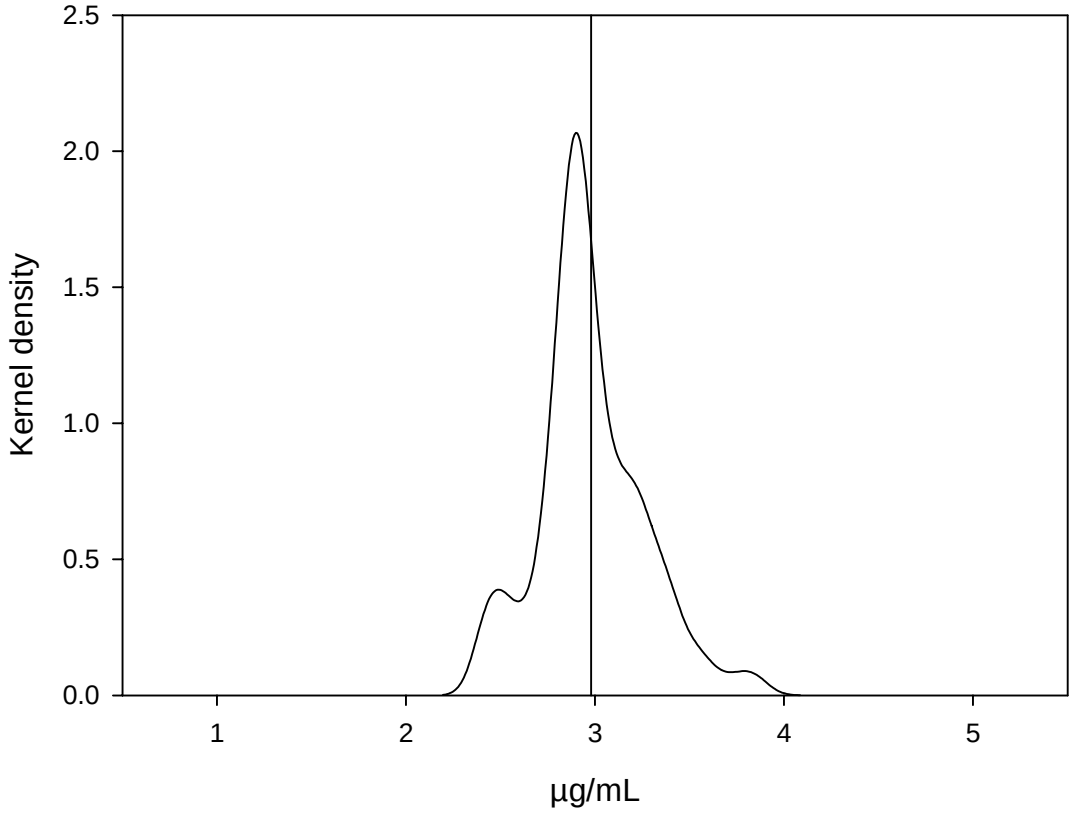


Figure 16: Kernel Density Plot for aflatoxin G_1 results:

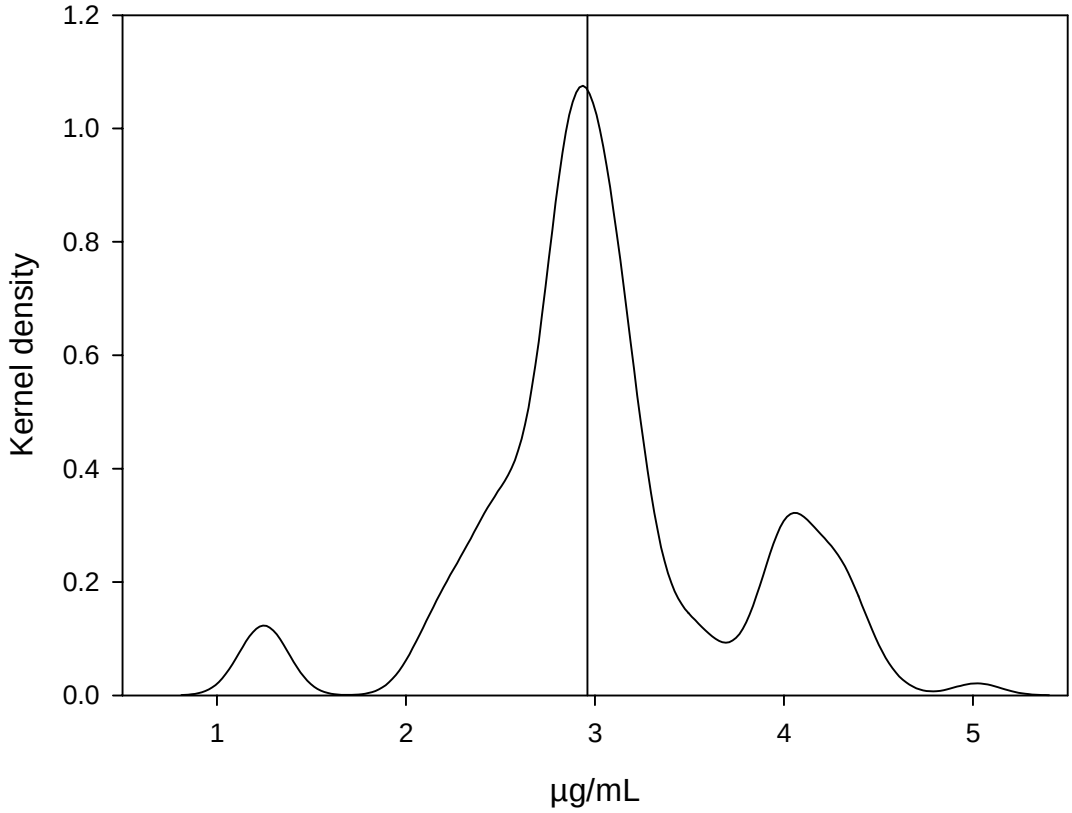
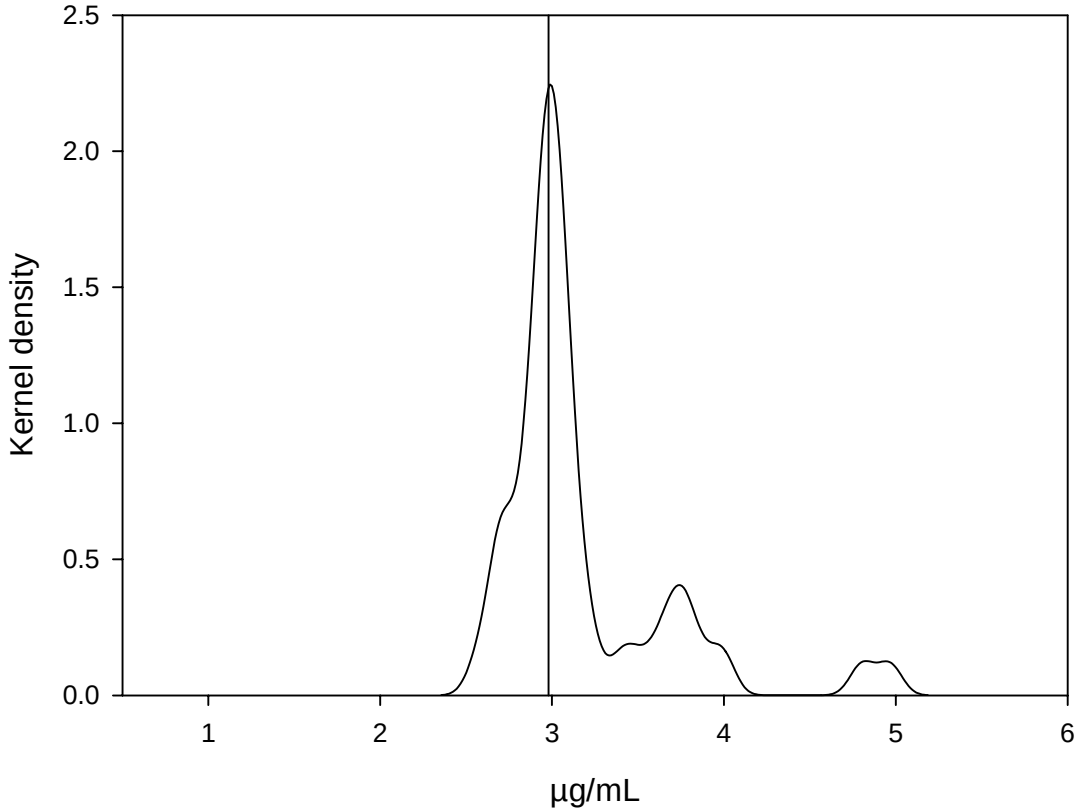


Figure 17: Kernel Density Plot for aflatoxin G_2 results:



Deviation of the mean result from the certified value

For aflatoxin B_1 determinations, 9 of the laboratories reported mean values that fell within the range (+/- MU) of the certified value. For 13 laboratories the neither the mean nor the standard deviation calculated from the reported measurements overlapped, while for 4 laboratories the standard deviation overlapped with the certification range, but the mean value was outside the certification range. No effect was observed depending on the source (commercially obtained or in-house prepared solutions).

For aflatoxin B_2 determinations, 8 of the laboratories reported mean values that fell within the range (+/- MU) of the certified value. For 13 laboratories the neither the mean nor the standard deviation calculated from the reported measurements overlapped, while for 5 laboratories the standard deviation overlapped with the certification range, but the mean value was outside the certification range. No effect was observed depending on the source (commercially obtained or in-house prepared solutions).

For aflatoxin G_1 determinations, 7 of the laboratories reported mean values that fell within the range (+/- MU) of the certified value. For 13 laboratories the neither the mean nor the standard deviation calculated from the reported measurements overlapped, while for 5 laboratories the standard deviation overlapped with the certification range, but the mean value was outside the certification range. No effect was observed depending on the source (commercially obtained or in-house prepared solutions).

For aflatoxin G_2 determinations, 9 of the laboratories reported mean values that fell within the range (+/- MU) of the certified value. For 8 laboratories the neither the mean nor the standard deviation calculated from the reported measurements overlapped, while for 8 laboratories the standard deviation overlapped with the certification range, but the mean value was outside the certification range. No effect was observed depending on the source (commercially obtained or in-house prepared solutions).

Deviation of the certified value for each aflatoxin, sorted by laboratories.

The pattern of deviation of the single aflatoxin results from the certified value as shown in figure 5, clearly show that deviations for AfG_1 very often co-occur with deviation of AfG_2, and that the deviations from the G aflatoxins are in general higher than those of the B aflatoxins. This reflects the higher instability of the G aflatoxins to alkaline ambient or non-acid washed glass ware compared to the B aflatoxins, due to the 2^{nd} lactone ring. Lowest deviations are observed for aflatoxin B_2, confirming its relative stability compared to the G aflatoxins as discussed above and to aflatoxin B_1 due to the lacking double bond at the 8, 9-position, which also contributes to the instability of the aflatoxins of type-$_1$. The origin of these effects can only be speculated and might occur during handling, storage or even analysis.
No difference can be seen in the distribution pattern of aflatoxins with a negative or positive deviation. This indicates that the overall effects contributing to the deviation pattern (whether they might have occurred during the handling or analysis of the reference solutions or the storage, handling or analysis of the in-house solutions) appear to be of the same nature for positive and negative deviations.

Furthermore, random deviations (between aflatoxins) without a significant bias from the certified values can only be observed for those cases where values close to the reference value were found for all aflatoxins.

Intermediate Reproducibility

The calculated intermediate reproducibility (Figures 6 - 9) is a parameter that is not directly linked to the quality of the in-house standards used for this exercise, rather than an indicator how well the precision of the methodology is under control. This is supported by the fact that the pattern and magnitude of this parameter is very similar for all aflatoxins under investigation. Three cut-off levels were chosen arbitrary to cluster laboratories. These are 5%, 10% and 15% and reflect on the basis of Horwitz for 3 µg/mL HORRAT values of 0.4, 0.7 and 1.1. Despite the fact that these values appear to be rather satisfactory, it must however be considered that HORRAT values were derived from collaborative trials for the determination of analytes in samples, requiring clean-up and other procedures and that the application of HORRAT therefore is very limited. As a result it is recommended that laboratories which have obtained values of more than 5% for this parameter, should undertake efforts to improve their day-to-day precision.

Deviation from the reference value

Different than the intermediate reproducibility, the deviation of the mean value from the reference value (Figure 10 - 13) is a parameter tightly linked to the quality of the in-house solutions used in this exercise as well as the above mentioned effects that can alter the aflatoxin content in solution. No general evaluation parameter exists for such a purpose and an arbitrary limit of 10% was chosen to make a ranking. As a result it is recommended that laboratories which have obtained values of more than 10% for this parameter, should undertake efforts to make sure that standards are prepared and checked on a regulatory basis for trueness of the calibrants. These improvement efforts should primarily focus on aflatoxin B_1, as being a single regulated aflatoxin, but also on aflatoxin G_1 as for this aflatoxin the highest deviations were observed.

Conclusion

It could be demonstrated that the utilization of calibrants as currently used in various national reference laboratories leads to a rather wide spread variation of results. This occurred despite the fact that most of the laboratories used standards that were photometrically determined for content. Few laboratories used commercially prepared standards and no difference was observed between commercially obtained or in-house prepared solutions.
This allows the conclusion, that the currently observed variability of analytical results in comparability tests, such as proficiency test schemes, for aflatoxins can be derived to a large degree from the calibration procedure and that calibrations procedures – as currently applied – are likely to add variances to the precision in the same magnitude as currently applied method variances derived from collaborative studies.
As a result the importance of the calibration must not be underestimated, as is of key importance for the correctness and thus the mutual acceptance of analytical results.

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Abstract

A proficiency test was conducted with 26 European National Reference Laboratories (NRLs) for mycotoxins. Test materials were aflatoxin stock solutions in acetonitrile with a previously certified content. Laboratories determined the aflatoxin content by liquid chromatography against their own standard solutions as reference by reverse-phase high-performance liquid-chromatography (RP-HPLC) with fluorescence detection. Laboratories used either commercially obtained standard solutions or gravimetrically prepared solutions from dry aflatoxin materials in combination with spectrophotometric confirmation of the content. The overall reproducibility values (RSDR) were 14.1%, 9.3%, 22.8% and 15.3% for Aflatoxin B1, B2, G1 and G2 respectively, reflecting the structure related sensibility of aflatoxins in solutions towards daylight and alkaline ambient. The precision figures reflect the variability of aflatoxin results that are solely related to currently applied calibration procedures.

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